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CHEMICAL AND BIOACTIVE CONSTITUENTS FROM *ZANTHOXYLUM SIMULANS*

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ABSTRACT.—Two new benzo[*c*]phenanthridine alkaloids, 6-methyldihydrochelerythrine [**1**] and 6-methylnorchelerythrine [**2**], together with 23 known compounds, were isolated from the root bark of *Zanthoxylum simulans*. Structures were elucidated by spectral analysis. Among them, the pyranoquinoline alkaloids, zanthosimuline [**3**] and huajiaosimuline [**4**], exhibited cytotoxic activity. In addition, compound **4** showed significant antiplatelet aggregation activity and induced terminal differentiation with cultured HL-60 cells.

In continuation of our chemical investigation of Formosan species of the genus *Zanthoxylum* (Rutaceae), we have isolated four new 2-quinolones and several known alkaloids from the root bark and root wood of *Zanthoxylum simulans* (1–3). Further examination of the C_6H_6-soluble part of a neutral fraction of root bark from this plant has resulted in the isolation of 25 compounds, two of which are new benzo[*c*]phenanthridine alkaloids, 6-methyldihydrochelerythrine [**1**] and 6-methylnorchelerythrine [**2**]. The cytotoxicity, antiplatelet aggregation, and induction of terminal cell differentiation by pyranoquinoline alkaloids are also described.

RESULTS AND DISCUSSION

Compound **1** was obtained as colorless needles, mp 203–205°, and its hreims exhibited a $[M]^+$ ion at *m/z* 363.1442, corresponding to the formula $C_{22}H_{21}NO_4$ (calcd 363.1471). The uv absorptions at 229, 256, 283, 325 sh, and 350 sh nm were similar to those of dihydrochelerythrine and were characteristic of a 2,3,7,8-oxygenated benzo[*c*]phenanthridine (4). The ^{1}H-nmr spectrum showed the presence of a *N*-methyl group [δ 2.60 (3H, s)] and two methoxy groups [δ 3.93, 3.94 (3H each, s)]; a methylenedioxy group [δ 6.05 (2H, s)]; two pairs of ortho-coupled aromatic protons [δ 6.94, 7.55 (1H each, d, J=8.5 Hz, H-9 and H-10) and δ 7.48, 7.73 (1H each, d, J=8.7

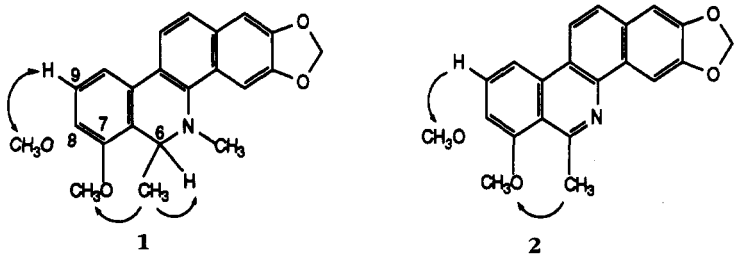


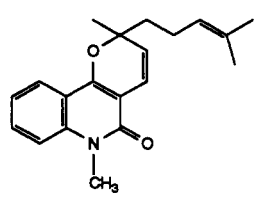
FIGURE 1. NOe Interactions Observed for **1** and **2**.

Hz, H-12 and H-11)], and two singlet aromatic protons at δ 7.12 (H-1) and 7.72 (H-4). Further analysis of the ^{1}H nmr spectrum of **1** revealed the presence of a mutually coupled benzylic proton at δ 4.58 (q, J=6.8 Hz, H-6) and a methyl signal at δ 1.12 (d, J=6.8 Hz). The relative locations of the methylenedioxy and methoxy groups were confirmed by enhancements observed between C-6 Me (δ 1.12) and H-6, 7-OMe and between the H-9 (δ 6.94) and 8-OMe in an nOe-DIF nmr experiment (Figure 1). These results indicated the locations of the two methoxy and methylenedioxy groups to be at C-7, C-8 and C-2, C-3, respectively. On the basis of the above data, structure **1** was assigned to 6-methyldihydrochelerythrine.

Compound **2** was isolated as colorless prisms, mp 198–200°. The molecular formula was determined as $C_{21}H_{17}NO_4$ by hrms. The uv absorption spectrum of **2** showed a close resemblance to that of norchelerythrine (5), thus suggesting a 2,3,7,8-oxygenated benzo[*c*]phenanthridine skeleton for compound **2**. The ^{1}H-nmr spectrum of **2** appeared as two pairs of ortho-coupled aromatic protons at δ 7.58, 8.31 (1H each, d, J=9.0 Hz) and at δ 7.79, 8.42 (1H each, d, J=9.1 Hz), which were attributed to H-9, H-10, and H-12, H-11, respectively. Two singlet signals at δ 7.25 and 8.76 were assigned to H-1 and H-4. In addition, one aryl methyl group [δ 3.31 (3H, s)], two methoxy groups [δ 4.02, 4.06 (3H each, s)] and a methylenedioxy group [δ 6.13 (s)] were observed. Furthermore, a nOe-DIF nmr experiment showed an enhancement of the signal at δ 4.02 (OMe-7) on irradiation at the frequency corresponding to the aryl methyl protons (Figure 1). In addition, the nOe enhancement between C-9 at δ 7.58 and the methoxy at δ 4.06 suggested the location of an aryl methyl and two methoxy groups at C-6, C-7, and C-8, respectively. The above data were in excellent accord with the structure of **2** being 6-methylnorchelerythrine.

Twenty-three known compounds were also isolated, comprising the benzo[*c*]phenanthridine alkaloids dihydrochelerythrine (6), norchelerythrine (7), bocconoline (4), 6-acetonyldihydrochelerythrine (8), oxychelerythrine (9), decarine (10), arnottianamide (11), chelerythrine (2), avicine (12); the quinoline alkaloids zanthosimuline (2), robustine (3), dictamnine (3), γ-fagarine (3), skimmianine (7), zanthobisquinolone (3), 8-methoxy-*N*-methylflindersine (13), *N*-methylflindersine (14), simulanoquinoline (2), toddaquinoline (7); the aporphine alkaloids (−)-*N*-acetylanonaine (15), (−)-*N*-acetylnornuciferine (15), liriodenine (16), and a lignan, hinokinin (17). These compounds were identified by comparisons of their tlc, ir, and/or mmp with corresponding authentic samples.

The cytotoxic activity of the new pyranoquinoline alkaloids, zanthosimuline [**3**] (2) and huajiaosimuline [**4**] (2) is summarized in Table 1. Compound **3** demonstrated a general cytotoxic response when evaluated with a variety of cultured human cancer cell lines and cultured P-388 cells using published methods of bioassay (18). Multidrug-resistant KB-VI cells demonstrated a sensitivity that was approximately equivalent to that observed with cultured KB cells. In the presence of vinblastine, activity was enhanced about 3-fold with KB-VI cells, suggesting reversal of the drug resistance of the



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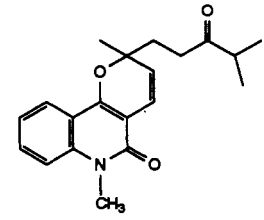

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TABLE 1. Cytotoxic Activity of Compounds **3** and **4**.

Compound	Cancer cell line (ED_{50}, μM)						
	BC-1	HT-1080	Lu1	Me1	Col2	KB	KB-V1 (+VLB)
3	28.8	28.8	28.8	28.4	30.4	15.2	6.5
4	>60	>60	>60	36.6	>60	46.4	4.0
	KB-V1 (−VLB)	P-388	A431	LNCaP	ZR-75-1	U373	
3	21.3	5.2	28.8	18.7	10.0	17.1	
4	24.3	9.8	>60	>60	11.1	>60	

Abbreviations: BC-1, human breast; HT-1080, human fibrosarcoma; Lu1, human lung; Mel, human melanoma; Col2, human colon; KB-V1, drug-resistant KB; VLB, vinblastine; A431, human squamous cell; LNCaP, human prostate; ZR-75-1, human breast; U373, human glioma.

phenotype [19]. Conversely, oxidation of the side-chain produced a more selective profile of cytotoxic activity, as exhibited by compound **4**. Of particular note, of the human tumor cell lines tested, greatest activity was observed with the estrogen receptor-positive breast cancer cells, ZR-75-1, and even more pronounced reversal of resistance to vinblastine was demonstrated with KB-VI cells.

In addition, prompted by the structural similarity of the compound **3** and **4** side-chains and vitamin D metabolites, studies were performed with cultured HL-60 cells. As summarized in Table 2, both compounds were able to induce the expression of cellular markers associated with cell differentiation, and compound **4** was more active than

TABLE 2. Induction of Cell Differentiation by Compounds **3** and **4** with Cultured HL-60 Cells.

Compound	% cell viability[1]	# of cells[2] ($X10^4$)	Conc. tested (μM)	Inhibition of [^{3}H] thymidine incorp. (%)	NBT reduction (%)	NSE activity (%)	SE activity (%)
3	9	1	64.6	Toxic	Toxic	Toxic	Toxic
	78	29	32.3	0.0±11.4	15.4±4.2	24.3±0.1	14.5±1.3
	93	51	16.2	0.0±10.2	9.0±1.0	7.4±0.3	6.5±0.3
	95	89	8.1	0.0±2.2	8.0±1.0	5.5±0.5	5.5±0.5
	98	143	4.0	0.0±0.5	3.5±0.5	4.5±0.5	5.0±0.0
4	85	16	61.5	31.6±1.8	53.1±6.0	48.9±2.9	5.0±1.0
	96	49	30.7	0.0±2.6	9.9±2.3	12.2±2.2	9.4±1.4
	98	94	15.4	0.0±9.0	8.0±2.0	5.5±0.5	8.0±0.9
	98	110	7.7	0.0±1.2	4.5±0.5	5.0±1.0	6.5±1.5
	97	106	3.8	0.0±3.8	5.5±0.5	5.0±0.0	5.5±0.5
$1\alpha,25(OH)_2D_3$	92	98	1	95.3±3.5	90.5±4.2	95.3±2.7	2.0±0.5
	95	125	0.1	92.0±3.0	80.0±1.3	90.1±0.6	2.0±0.0
	97	139	0.01	58.3±4.5	73.3±8.3	68.0±4.9	4.0±0.6
	99	145	0.001	32.3±8.0	28.5±2.9	14.0±0.9	4.6±0.5
	98	128	0.0001	6.0±0.7	8.8±5.7	2.5±0.7	5.0±0.8

[1]The cell viability after 4 days of incubation with compounds was determined by trypan blue exclusion. [% cell viability=viable cells/(viable cells+dead cells)×100].

[2]The starting cell number (day 0) was 20×10^4 cells/ml; the reported values are the number of cells observed after 4 days of incubation.

compound **3** in this capacity. However, relative to the positive control compound, 1α,25-dihydroxyvitamin D_3, both compounds are regarded as weakly active.

Finally, zanthosimuline [**3**], huajiaosimuline [**4**], simulanoquinoline, and toddaquinoline were evaluated for antiplatelet aggregation activity (1). The aggregation of rabbit platelets induced by arachidonic acid (100 μM), collagen (10 μg/ml), or PAF (2 ng/ml) was inhibited by 100%, 83.9%, and 100%, respectively, in the presence of huajiaosimuline [**4**] at 100 μg/ml. Compound **3**, simulanoquinoline, and toddaquinoline were not active at this concentration.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All mps were determined on a Yanaco micro-melting point apparatus and are uncorrected. Mass spectra were obtained on a Hitachi RMU-6E spectrometer. ^{1}H-Nmr and nOe-DIF spectra were measured on either a Varian Gemini 200 or JEOL-GSX-500 spectrometer and are given in ppm (δ) downfield from internal TMS. Uv spectra were obtained on a Hitachi U-200 spectrophotometer, and ir spectra were recorded on a Hitachi 260-30 (KBr) spectrophotometer.

PLANT MATERIAL.—The root bark of *Z. simulans* was collected from Taichung Hsien, Taiwan, in October 1985. A voucher sample is deposited in the Herbarium of Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

EXTRACTION AND ISOLATION.—The C_6H_6-soluble part (34.03 g) of a neutral fraction derived from the MeOH extract of the root bark of *Z. simulans*, as described in a previous paper (2), was chromatographed over Si gel and eluted with a gradient of $CHCl_3$ and MeOH to give 11 fractions. Fraction 1 (0.15 g) was rechromatographed on Si gel, using a gradient of C_6H_6/EtOAc as eluent and recrystallized with $CHCl_3$/MeOH to yield dihydrochelerythrine (2.3 mg), norchelerythrine (9.6 mg), and bocconoline (4.7 mg). Further, the mother liquors of dihydrochelerythrine, norchelerythrine, and bocconoline were purified by prep. tlc (*n*-hexane-EtOAc, 5:1) to afford 6-methyldihydrochelerythrine (1.9 mg), hinokinin (3.1 mg), 6-methylnorchelerythrine (1.1 mg), robustine (1.1 mg), 8-methoxy-*N*-methylflindersine (10.4 mg), and 6-acetonyldihydrochelerythrine (3.0 mg). Fraction 2 (0.48 g) was washed with Et_2O then purified by recrystallization to give (−)-*N*-acetylanonaine (30 mg). The eluate was concentrated and subjected to Si gel chromatography and eluted with C_6H_6 and C_6H_6/EtOAc gradients, then purified by prep. tlc (*n*-hexane-EtOAc, 5:1) to yield hinokinin (2.9 mg), dihydrochelerythrine (2.1 mg), dictamnine (2.2 mg), zanthobisquinolone (5.3 mg), (−)-*N*-acetylanonaine (5.0 mg), norchelerythrine (9.9 mg), 6-acetonyldihydrochelerythrine (5.0 mg), bocconoline (3.8 mg), and γ-fagarine (3.0 mg), successively. Fraction 3 (0.39 g) was separated on a Si gel column and eluted with C_6H_6-EtOAc (10:1 and 5:1) to afford dictamnine (1.2 mg), zanthobisquinolone (2.6 mg), norchelerythrine (9.9 mg), 6-acetonyldihydrochelerythrine (5.0 mg), and bocconoline (2.5 mg), successively. Fractions 4–8 were washed with MeOH individually to give (−)-*N*-acetylanonaine (3.76 g). The eluates from fractions 4–8 were combined and subjected to Si gel column chromatography using C_6H_6 and C_6H_6/EtOAc gradients as eluent to yield robustine (1.3 mg), *N*-methylflindersine (3.2 mg), dihydrochelerythrine (5.6 mg), norchelerythrine (20 mg), bocconoline (11.6 mg), zanthosimuline (18.9 mg), 8-methoxy-*N*-methylflindersine (9.6 mg), zanthobisquinolone (7.1 mg), (−)-*N*-acetylnornuciferine (2.3 mg), and γ-fagarine (2.0 mg), respectively. Fraction 9 (5.23 g) was separated by cc on Si gel to give (−)-*N*-acetylanonaine (230 mg), simulanoquinoline (9.0 mg), skimmianine (13.6 mg), oxychelerythrine (3.2 mg) and toddaquinoline (3.3 mg), respectively, from C_6H_6-EtOAc (5:1). Fraction 10 (3.01 g) was rechromatographed on Si gel, using $CHCl_3$ and $CHCl_3$-EtOAc (5:1 and 3:1) as eluent to obtain (−)-*N*-acetylanonaine (34.1 mg), liriodenine (1.5 mg), and arnottianamide (4.7 mg), respectively. Fraction 11 (0.43 g) was further chromatographed over Si gel and eluted with $CHCl_3$-MeOH (25:1 and 10:1) to yield arnottianamide (5.6 mg), decarine (3.7 mg), avicine (2.7 mg), and chelerythrine (2.1 mg).

6-METHYLDIHYDROCHELERYTHRINE [**1**].—Colorless needles (MeOH), mp 203–205°; hreims, found M^+ 363.1442, $C_{22}H_{21}NO_4$, requires 363.1471; uv λ max (EtOH) (log ε) 229 (4.74), 256 (4.39), 283 (4.79), 325 sh (4.30), 350 sh (3.62) nm; ir ν max (KBr) 2920, 2840, 1490, 1460, 1410, 1260, 1245, 1100, 1030, 940, 860, 800 cm^{-1}; ^{1}H nmr δ 1.12 (3H, d, J=6.8 Hz, Me-6), 2.60 (3H, s, N-Me), 3.93, 3.94 (3H each, s, OMe-7 and OMe-8), 4.58 (1H, q, J=6.8 Hz, H-6), 6.05 (2H, s, OCH_2O), 6.94 (1H, d, J=8.5 Hz, H-9), 7.12 (1H, s, H-1), 7.48 (1H, d, J=8.7 Hz, H-12), 7.55 (1H, d, J=8.5 Hz, H-10), 7.72 (1H, s, H-4), 7.73 (1H, d, J=8.7 Hz, H-11); eims *m/z* 363 (M^+, 36), 349 (26), 348 (100), 333 (12), 332 (14), 318 (11), 304 (10), 290 (17).

6-METHYLNORCHELERYTHRINE [**2**].—Colorless prisms ($CHCl_3$/MeOH), mp 198–200°; hreims *m/z*,

found M^+ 347.1163, $C_{21}H_{17}NO_4$, requires 347.1158; uv λ max (log ε) 210 (4.37), 243 (4.61), 255 (4.45), 279 (4.72), 323 (4.19), 363 (3.59), 380 (3.56) nm; ir ν max 2910, 2850, 1565, 1455, 1280, 1250, 1030, 950, 880 cm^{-1}; ^1H nmr δ 3.31 (3H, s, Me-6), 4.02 and 4.06 (3H each, s, OMe-7 and OMe-8), 6.13 (2H, s, OCH_2O), 7.25 (1H, s, H-1), 7.58 (1H, d, J=9.0 Hz, H-9), 7.79 (1H, d, J=9.1 Hz, H-12), 8.31 (1H, d, J=9.0 Hz, H-10), 8.42 (1H, d, J=9.1 Hz, H-11), 8.76 (1H, s, H-4); eims *m/z* 347 (M^+, 100), 332 (23), 304 (32), 289 (23).

ANALYSIS OF CYTOTOXIC POTENTIAL.—Compounds were tested for cytotoxic potential utilized cultured cell lines as described previously (18). In each case, complete dose-response data were generated utilizing at least five concentrations of test substances evaluated in duplicate.

HL-60 CELL DIFFERENTIATION ASSAYS.—Procedures were similar to those described previously (20,21). HL-60 (human promyelocytic leukemia) cells were maintained in continuous suspension culture in RPMI 1640 medium (GIBCO) supplemented with 5% heat-inactivated calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml at 37° in a humidified atmosphere of 5% CO_2 in air. HL-60 cells were passed at a density of 2×10^5 cells/ml twice a week.

To test the potency of agents to induce cell differentiation, HL-60 cells in the log phase (approximately 1×10^6 cells/ml) were diluted (1.2×10^5 cells/ml) and preincubated for 18 h in 24-well tissue culture plates. Samples dissolved in dimethylsulfoxide (DMSO) were then added, keeping the final DMSO concentration at 0.1%. Control cultures were treated with the same concentration of DMSO. After 4 days of incubation, the cells were analyzed to determine the percentage exhibiting functional and enzymatic markers of differentiated cells, as judged by the three methods described below.

Nitroblue tetrazolium (NBT) reduction.—A 1:1 v/v mixture of a cell suspension (10^6 cells) and freshly prepared TPA/NBT solution (phosphate buffered saline solution containing 2 mg/ml of NBT and 5 μg/ml of TPA) was incubated for 1 h at 37°. Positive cells are able to reduce NBT yielding intracellular black-blue formazan deposits and this can be determined by microscopic examination. The results are expressed as a percentage of NBT-positive cells.

Nonspecific/specific acid esterase activity.—Assays for α-naphthyl acetate esterase and naphthol AS-D chloroacetate esterase were performed using cytochemical kits from Sigma (91-A and 91-C).

[^{3}H]Thymidine incorporation.—Sample-treated cells (100 μl) were removed and placed into 96-well plates and 0.5 μCi/ml [^{3}H]thymidine (specific activity, 65 Ci/mmol) was added. After 16 h of further incubation, cells were harvested using a Skatron cell harvester and counted by liquid scintillation spectroscopy. The percentage of [^{3}H]thymidine incorporation per 10^6 cells was calculated by dividing sample DPM by DMSO control DPM.

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LITERATURE CITED

1. F.N. Ko, I.S. Chen, S.J. Wu, L.G. Lee, T.F. Haung, and C.M. Teng, *Biochim. Biophys. Acta,* **1052**, 360 (1990).
2. S.J. Wu and I.S. Chen, *Phytochemistry,* **34**, 1659 (1993).
3. I.S. Chen, S.J. Wu, Y.C. Lin, I.L. Tsai, H. Seki, F.N. Ko, and C.M. Teng, *Phytochemistry,* **36**, 237 (1994).
4. H. Ishii, T. Ishikawa, K. Hosoya, and N. Takao, *Chem. Pharm. Bull.,* **26**, 166 (1978).
5. A.W. Sangster and K.L. Stuart, *Chem. Rev.,* **65**, 69 (1965).
6. F. Fish, A.I. Gray, and P.G. Waterman, *Planta Med.,* **25**, 282 (1974).
7. I.S. Chen, I.L. Tsai, S.J. Wu, W.S. Sheen, T. Ishikawa, and H. Ishii, *Phytochemistry,* **34**, 1449 (1993).
8. D.B. Maclean, D.E.F. Gracey, J.K. Saunders, R. Rodrigo, and R.H.F. Manske, *Can. J. Chem.,* **47**, 1951 (1969).
9. M. Onda, K. Abe, K. Yonezawa, N. Esumi, and T. Suzuki, *Chem. Pharm. Bull.,* **18**, 1435 (1970).
10. P.G. Waterman, *Phytochemistry,* **14**, 843 (1975).
11. H. Ishii, T. Ishikawa, S.T. Lu, and I.S. Chen, *Tetrahedron Lett.,* 1023 (1976).
12. H. Ishii, T. Ishikawa, Y.I. Ichikawa, M. Sakamoto, M. Ishikawa, and T. Takahashi, *Chem. Pharm. Bull.,* **32**, 2984 (1984).
13. M.S. Hifnawy, J. Vaquette, T. Sévenet, J.L. Pousset, and A. Cavé, *Phytochemistry,* **16**, 1035 (1977).
14. H. Ishii, J.I. Kobayashi, and T. Ishikawa, *Tetrahedron Lett.,* **32**, 6907 (1991).
15. C.D. Hufford, *Phytochemistry,* **15**, 1169 (1976).

16. C.L. Chen, H.M. Chang, and E.B. Cowling, *Phytochemistry,* **15**, 547 (1976).
17. Y.L Huang, C.C. Chen, and J.C. Ou, *Planta Med.,* **58**, 473 (1992).
18. K. Likhitwitayawuid, C.K. Angerhofer, G.A. Cordell, J.M. Pezzuto, and N. Ruangrungsi, *J. Nat. Prod.,* **56**, 30 (1993).
19. A. Somanabandhu, S. Nitayangkura, C. Mahidol, S. Ruchirawat, K. Likhitwitayawuid, H.L. Shieh, H. Chai, J.M. Pezzuto, and G.A. Cordell, *J. Nat. Prod.,* **56**, 233 (1993).
20. V.K. Ostrem, Y. Tanaka, J. Prahl, H.F. Deluca, and N. Ikekawa, *Proc. Natl. Acad. Sci. USA,* **84**, 2610 (1987).
21. H. Tanaka, E. Abe, C. Miyaura, T. Kuribayashi, K. Konno, Y. Nishi, and T. Suda, *Biochem. J.,* **204**, 713 (1982).

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